Ethanolic extract and ethyl acetate fraction of *Coutoubea spicata* attenuate hyperglycemia, oxidative stress and muscle damage in alloxan-induced diabetic rats subjected to resistance exercise training program

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Ethanolic extract and ethyl acetate fraction of *Coutoubea spicata* attenuate hyperglycemia, oxidative stress and muscle damage in alloxan-induced diabetic rats subjected to resistance exercise training program


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ABSTRACT

Gentianaceae family (such as *Coutoubea spicata* – *C. spicata*) contains iridoids and flavonoids with antidiabetic properties. However, there is no information available about the antidiabetic effects of *C. spicata* when associated with resistance exercise training (RET). The study evaluated the effects of the ethanolic extract (EE) and ethyl acetate fraction (EAF) of *C. spicata* on biochemical markers, muscle damage and oxidative stress in diabetic rats submitted to RET. Alloxan-induced diabetic rats were distributed into 4 groups (n=8, each) treated with distilled water (TD), EE (TE), EAF (TF) or metformin (TM) and submitted to RET. Two groups without the disease (n = 8, each) (sedentary control [SC] and trained control [TC]), as well as a sedentary diabetic (SD, n=8) were included. Body weight and glycemia were evaluated weekly. After 30 days, lipid/lipoprotein profile, aspartate aminotransferase (AST), alanine aminotransferase (ALT), muscle damage [(creatine kinase (CK) and lactate dehydrogenase (LDH)] and oxidative stress [malondialdehyde (MDA), sulphydryl groups (SH) and ferric reducing antioxidant power (FRAP)] were evaluated. MDA and SH for pancreas, liver, heart and muscle were evaluated. *C. spicata* extract and fraction associated with RET recovered body weight, and reduced glycemia, muscle damage (CK: 36,83%; 21,45% and LDH: 49,83%; 68,55%) and LDL-cholesterol (70,63%; 59,18%), and improved redox status (MDA: 50,33%; 39,74% and SH: 53,97%; 76,41%), respectively, when compared to TD group. *C. spicata* plus RET promoted anti-hyperglycemic, lipid-reducing and antioxidant effects in diabetic rats.

Novelty

*C. spicata* presents anti-hyperglycemic and lipid-lowering effects potentiated by RET.

*C. spicata* reduces muscle injury and increases antioxidant defense.

**Keywords:** *Coutoubea spicata*, anti-hyperglycemic, resistance exercise, dyslipidemia, antioxidant, natural products
1. Introduction

Diabetes mellitus (DM) is an important public health problem with growing incidence and is expected to reach 693 million people worldwide by 2045. Hyperglycemia, when uncontrolled, can cause damage to various organs, compromising quality of life and leading to life-threatening health complications such as cardiovascular diseases, neuropathy, nephropathy and retinopathy (IDF 2017; Palacios et al. 2019).

Hyperglycemia is closely related to oxidative stress, as it causes tissue damage and activates important inflammatory pathways, while cellular survival pathways activated by reactive oxygen species (ROS) are attributed to this condition (Cheng et al. 2011). ROS-forming pathways such as glucose autoxidation, increased mitochondrial electron transport chain activity, advanced glycation end products, and protein kinase C activation are well described in diabetes (Brownlee 2005; Giacco and Brownlee 2010; Shah and Brownlee 2016; Ighodaro 2018).

The treatment of diabetes involves pharmacological and non-pharmacological strategies. For non-pharmacological treatment, changes in lifestyle such as the incorporation of physical exercise can promote benefits such as improved blood glucose and lipid/lipoprotein profile, and aerobic capacity (Wu et al. 2019; SBD 2017). Recent studies have shown that resistance exercise training program (RET) increases glucose uptake in the skeletal muscle by stimulating the translocation of GLUT-4 to the sarcoplasmic membrane, which is characterized by being insulin-independent, and post-translational modifications of proteins involved in exercise-induced fatty acid uptake and oxidation (Böhm et al. 2016; Santos et al. 2017; Widmann et al. 2019). During the RET the skeletal muscle undergoes momentary states of ischemia/reperfusion, being one of the mechanisms responsible for the ROS increase as well consequent muscular oxidative damage (Cruzat et al. 2007; Petry et al. 2010). However, the relationship between exercise and oxidative stress is extremely complex, depending on the exercise type, intensity and duration (Pingitore et al. 2015).

Another widely used alternative treatment for DM is medicinal plants, which are traditionally used worldwide and present as a safe, reliable and economical alternative (Bharti et al. 2018; Gupta 2018).

*Coutoubea spicata* (*C. spicata*) belongs to the Gentianaceae family, and gentiopicrin and swertiamarin are among the main phytochemical constituents already identified, which are secoiridoid glycosides responsible for the bitter taste, in addition to compounds of the group of flavonoids among them clovin, robinin and their esters such as p-coumaric acid (Schaufelberger et al. 1987).
The anti-hyperglycemic effect already described in these species of plants is due to the presence of compounds swertiamarin, swertisin and lupeol (Ghazanfar et al. 2017). This effect can be explained, at least in part by cytoprotective effects on pancreatic β cells, associated with a potentiating effect on insulin secretion, insulin signaling pathway improvement and inhibition of hepatic gluconeogenesis (Patel and Mishra 2011; Leong et al. 2016; Patel et al. 2018).

The aim of this study was to evaluate the effects of the ethanolic extract and the ethyl acetate fraction of *C. spicata* on body weight, biochemical and muscle damage markers in alloxan-induced diabetic rats submitted to RET. Due to the reported properties to Gentianaceae family, our hypothesis is anti-hyperglycemic effect of *C. spicata* is enhanced by the physical training, accompanied by reduction of oxidative stress.

2. Material and Methods

Plant material: collection, processing and extraction

Aerial parts of *C. spicata* were collected in March 2011 in the municipality of Japaratuba, State of Sergipe, Brazil (10°32’4.49’S and 36°53’57’W). The plant material was identified by botanist PhD Ana Paula do Nascimento Prata and a specimen was deposited under registration number ASE 25.136 (SISGEN A6AC079) in the Herbarium of the Federal University of Sergipe, São Cristóvão, Sergipe, Brazil.

A total of 1.2 kg of aerial parts of *C. spicata* were dried at room temperature, reduced to powder and subjected to maceration with 95% ethanol for 5 days. After-wards, the material was filtered and concentrated in a rotatory evaporator under reduced pressure at 45°C to give 424 g of the ethanolic extract (yield of 9.53%). A portion of ethanolic extract (252.0 g) was dissolved in methanol: water (2:3) and subjected to liquid-liquid extraction with organic solvents to obtain hexane (28.5 g, yield of 11.31 %), chloroform (51.2 g, yield of 20.32 %), ethyl acetate (45.8 g, yield of 18.17 %) and hy-dromethanol (123.4 g, yield of 48.97 %) fractions. Ethanolic extract (EE) and ethyl acetate fraction (EAF) were used in the *in vivo* assay.

Fifty-six male 3-month-old Wistar rats weighing 250-300 g were obtained of the Nucleus of Research in Intracellular Signaling (NUPESIN) of the Federal University of Sergipe (UFS). Animals were housed in cages at controlled temperature (22 ± 3 °C) with 12-h light / dark cycle (lights on, 6:00 a.m. - 6:00 p.m.) with free access to rodent feed (NuviLAB) and water *ad libitum*. All procedures described in this study were approved by the UFS Animal Research Ethics Committee (CEPA Protocol 26/18).
Diabetes mellitus induction

Experimental DM was induced by the administration of a 2% aqueous alloxan solution (Alloxan monohydrate A7413 - Sigma St. Louis USA) intraperitoneally injected in 40 animals at a single dose of 150 mg/kg body weight (Lerco et al. 2003). After a week adaptation, the animals were fasted for 24 hours to improve the sensitivity and diabetogenic action of the drug, with ad libitum water supply. Control animals (not diabetes, n = 16) received the same water volume. After 30 minutes of alloxan administration, feed was offered to all groups to prevent hypoglycemia. Blood glucose was evaluated after 72 h of induction and blood was collected by caudal puncture by means of lancets for blood glucose test, which was determined by means of an Accu-Chek Go glycosimeter (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). Animals with fasting blood glucose of 200 mg/dL or greater were included in the study. After confirmation of diabetes induction, animals were randomly distributed. Treatment and the RET protocol were performed, as shown in Figure 1.

Experimental groups: body weight and blood glucose (caudal puncture) were monitored once a week. Treatment and RET protocol were performed three times a week. For this, animals received vehicle (distilled water) or treatment with volume of 0.5ml/100 g of body weight.

Animals were distributed into 7 groups (n = 8): group 1) Sedentary Control (SC): healthy and sedentary animals treated with vehicle (distilled water orally administrated) + electrostimulation with animal suspended in the apparatus; group 2) Sedentary Diabetic (SD): diabetic animals treated with vehicle + electrostimulation with animal suspended in the apparatus; group 3) Trained Control (TC): healthy animals treated with vehicle and submitted to RET; group 4) Trained Diabetic (TD): diabetic animals treated with vehicle and submitted to RET; group 5) Trained Extract (TE): diabetic animals treated with C. spicata ethanolic extract (100 mg/kg, orally administrated) and submitted to RET; group 6) Trained Fraction (TF): diabetic animals treated with C. spicata ethyl acetate fraction (100 mg/kg, orally administrated) and submitted to RET; group 7) Trained Metformin (TM): diabetic animals treated with metformin (100 mg/kg, orally administrated) and submitted to RET. Groups are shown in Table 1.

For the RET protocol, an apparatus of the model proposed by Tamaki et al. (1992) and adapted by Santos et al. (2014) was prepared. The apparatus consists of an electronic model that emits electric current of 20 V of 0.3 s of duration, and 3 s of intervals applied in the tail.
All animals underwent a period of 1-week adaptation, where they received only electrical stimulation. The RET protocol was composed of 3 sets of 10 repetitions, with rest intervals of 60 s, and intensity of 65 % of the established load by means of the one repetition test (1 RM) performed 3 times a week, every other day. Animals were stimulated to perform the series by applying electrical stimuli using electrodes (ValuTrode, Model CF3200, Axelgaard, Fallbrook, CA, USA) attached to the tail and connected to an electrostimulator (BIOSET, Physiotonus Four, Model 3050, Rio Claro, SP, Brazil). The electrostimulation used is according to Barauna et al. (2005), so that stress is only momentary and does not interfere with stress-related pathways (release of catecholamines). Training load and intensity for groups submitted to RET were readjusted every 2 weeks by a new 1 RM test Table 2.

Animals belonging to the sedentary groups (SC and SD) were manipulated and fixed to the apparatus 3 times a week, on alternate days, with only electrostimulation, which did not generate adaptation to training.

Sample collection

After 30 days, animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Then, blood and tissues (pancreas, heart, liver and gastrocnemius muscle) were collected, weighed and properly stored for further analysis. The animals were sacrificed via exsanguination under anesthesia.

Determination of serum biochemical markers

Blood was centrifuged at 800 g for 15 minutes at 4 °C and serum stored at -80 °C. In serum, triglycerides, total cholesterol, LDL, HDL, ALT, AST, CK, LDH and uric acid concentrations were determined according to manufacturer's procedures (Labtest, Lagoa Santa, Minas Gerais, Brazil). Oxidative stress markers were also evaluated. Lipoperoxidation was determined by the measurement of thiobarbituric acid reactive substances (TBARS) according to method described by Lapenna et al. (2001). The determination of sulfhydryl groups (SH) was performed according to methodology described by Faure and Lafond (1995). The antioxidant capacity of the serum was measured by the Ferric Reducing Antioxidant Power (FRAP) method, according to protocol described by Pulido et al. (2000).

Determination of oxidative stress markers in tissues

To prepare homogenates, organs were removed and washed thrice in potassium chloride solution (1.15 % KCl), being then homogenized (1: 5 w / v) with solution containing
KCl, phenylmethylsulfonyl fluoride (PMSF 100 m.mol⁻¹) and Triton solution (10 %). Homogenates were centrifuged at 3000 g for 10 minutes at 4 °C and the supernatant stored at -80 °C for the determination of oxidative stress markers (TBARS and SH) according to methodology described in the previous item. Results were expressed as g of tissue.

**Statistical analysis**

Results are represented as mean ± standard error of the mean (SEM). Significant statistical difference was adopted among samples for p <0.05. All analyses were performed in triplicate. After data normality evaluation using the Shapiro Wilk test, they were statistically evaluated between groups through analysis of variance (One-way ANOVA) and repeated measures ANOVA for body weight and glucose followed by Bonferroni post-test. For this, the Graph Pad Prism statistical software version 7.0 was used.

3. Results

Diabetic animals presented with polyphagia, polydipsia and polyuria (data not shown) after the administration of alloxan and indicate time when symptoms began. These symptoms persisted until the end of the experiment. Figure 2 shows the results of body weight and blood glucose of groups during the experimental period. Differently from control animals (SC and TC), which maintained weight gain, sedentary diabetic animals (SD) presented marked body weight loss when compared to the other diabetic groups (Figure 2A). However, the association of RET program to treatment with *C. spicata* extract or fraction promoted body weight recovery from the tenth day of intervention.

At the end of the experimental period, the blood glucose levels of SD and TD rats were significantly higher than control rats (SC and TC) (Figure 2B). There was also reduction in the blood glucose levels of groups treated with plant extract (TE and TF) when compared to TD group. The reduction of the blood glucose concentration in TD, TE, TF and TM groups in relation to SD was accompanied by body weight gain recovery.

Table 3 presents the results for serum markers evaluated after the treatment period. Diabetic animals presented significant changes in the lipid/lipoprotein profile when compared to sedentary control animals. Concentrations of total cholesterol and fractions were reduced in the SD group when compared to the SC group. In addition, significant increase in triglyceride concentrations was observed in SD compared to SC animals. Moreover, the TD group presented significant increase in the concentrations of triglycerides, total cholesterol and fractions when compared to TC. The TD group showed significant increase in the total
cholesterol (~121 %) and LDL cholesterol concentrations (113%) when compared to the SD group. On the other hand, treatment with *C. spicata* extract and fraction reduced serum total cholesterol (~79 % and ~65.8 %) and LDL cholesterol concentrations (~70.6 % and ~59 %), respectively, when compared to the TD group (Table 3).

Animals in the SD group had ~296 % and ~107.8 % higher CK and AST concentrations, respectively, when compared to SC animals. The TD group presented significant increase in CK (~63.4 %) and AST concentrations (~43.6 %) when compared to the TC group. In contrast, LDH levels were significantly lower in TE, TF and TM groups when compared to the TD group. However, reduction in CK concentrations was observed only for TE and TM groups when compared to TD. Regarding uric acid concentrations, only TE group presented lower values when compared to TD and SD groups. No alterations were observed in ALT concentrations, regardless of group.

In relation to oxidative stress markers (Table 3), SD group had higher MDA (~293 %), SH (~84 %) and FRAP (~77 %) when compared to SC group. TD group demonstrated significant increase in MDA (~142.5 %) and FRAP (~70 %) when compared to TC group. On the other hand, groups that received treatment with plant extract or fraction associated to RET presented significant reduction in MDA (~50.3 % and ~39.7 %) and SH concentrations (~53.9 % and ~76.4 %) when compared to TD and SD groups, respectively.

Changes in oxidative stress markers were also observed in tissues evaluated. Figure 3 shows the MDA and SH results of pancreas, where significant increase of MDA in the SD group when compared to the SC group can be observed. On the other hand, treatment with *C. spicata* associated with RET promoted significant reduction in MDA concentrations in relation to SD and TD groups. Regarding SH concentrations, TD group presented significantly higher values when compared to TC group. Likewise, plant extract and fraction associated with physical training significantly reduced SH concentrations in relation to TD group, while TE group also showed lower SH concentrations in relation to SD group.

In the hepatic tissue as shown in Figure 4, increase in MDA and SH concentrations was observed in SD and TD groups when compared to SC and TC groups, respectively. RET associated with *C. spicata* extract or fraction altered the oxidative profile of the hepatic tissue, reducing the MDA marker. Regarding SH marker, treatment with extract or fraction significantly reduced the concentrations of this marker in relation to untreated diabetic groups (SD and TD).

The results of MDA and SH in cardiac tissue are shown in Figure 5. Diabetes increased MDA concentration in SD group. However, no differences were observed in these
values for groups treated with C. spicata, although SH values were reduced when compared with TD.

The MDA and SH concentrations in skeletal muscle tissue were significantly higher in SD group than in SC group. When comparing TD and TC groups, an increase only in MDA concentration was observed. Furthermore, the association of C. spicata with RET promoted significant reduction in MDA and SH concentrations in relation to diabetic animals (SD and TD) (Figure 6).

The combination of metformin with RET improved hyperglycemia and, consequently, promoted body weight gain recovery in diabetic animals, and such positive results may be due, at least in part, to the reduction of oxidative stress in tissues, confirmed by the lower MDA and SH values.

4. Discussion

The present study shows the effects of C. spicata extract and ethyl acetate fraction associated with RET in alloxan-induced diabetic rats on body weight, blood glucose control and attenuation of metabolic markers and oxidative stress in target tissues. In this way, this work contributes with scientific evidences that justify the use of C. spicata associated with RET to treat diabetes.

Groups treated with C. spicata extract or fraction and group treated with metformin showed significant body weight reduction in the first days of treatment; however, recovery to baseline values was optimized in TE and TF groups. According to Dhanavathy (2015), the marked body weight loss is due to dehydration and, mainly, muscular proteolysis, which is caused by the catabolism of tissue proteins due to the strong alterations in the carbohydrate metabolism, in addition to inhibition of protein synthesis induced by insulin resistance (Haber et al. 2001).

Both C. spicata extract and fraction showed anti-hyperglycemic effect throughout the treatment, since reduction of blood glucose levels was observed in TE and TF experimental groups. Although RET promoted reduction in blood glucose concentrations in isolation, it was even greater with the combination of different types of treatment.

Therefore, this is the first study to show the anti-hyperglycemic and antioxidant effects of C. spicata in alloxan-induced type-1 diabetic animals (DM 1) and submitted to RET, whose protocol is supported by literature in relation to the number of series. Turner et al. (2015) obtained better results with acute RET in the glycemic control of DM 1 patients
submitted to three series of resistance exercise against a single series or two series, although in none of the three situations, resistance exercise caused hypoglycemia during recovery.

It is known that RET performed at intensities of 60 % of 1 RM is capable of promoting structural, functional and metabolic changes in skeletal muscles and, at higher intensities, may cause greater changes (Boulé et al. 2001) including increased cell glucose uptake and increased activity of glycogen synthetase enzyme and GLUT 4 receptor expression. In a study by Yardley et al. (2013), moderate to high resistance exercise was better associated with glycemic stability in DM 1 when compared to aerobic exercise due to the greater "overload" of glycolytic stability.

The above effects seem to have been favored by *C. spicata*, since a phytochemical study previously conducted by the research group with *C. spicata* extract and fraction identified three classes of compounds, such as the glycosylated quercetin (clovin), robinin (kaempferol) and their esters with p-coumaric acid; deoxyloganic acid and iridoids (gentiopicrin and swertiamarin). The latter, in particular, are described as phytochemical markers for the Gentianaceae family and responsible for the bitter taste (Schaufelberger et al. 1987). Although anti-hyperglycemic effect attributed to swertiamarin has been observed in a study conducted by Dhanavathy (2015), in addition to the anti-catabolic action, preventing body weight loss, the active compounds present in *C. spicata* perform functions in an isolated or synergistic way with other minor components.

Thus, the possible mechanisms by which *C. spicata* exerted its anti-hyperglycemic effects may be explained in part by the ability to partially recover damaged β-cells, demonstrating its cytoprotective effects already described for swertiamarin (Dhanavathy 2015), which would increase insulin secretion, as well as by modulating important signaling pathways, such as insulin receptor (IR) and PI3K (Leong et al. 2016). However, the role of quercetin in modulating carbohydrate and lipid metabolism by the Akt/PI3K pathway, attenuating hyperglycemia, dyslipidemia and insulin resistance has been largely described (Alam et al. 2014; Peng et al. 2017; Senyigit et al. 2018). Thus, the anti-hyperglycemic effects of *C. spicata* can be explained by the synergism among compounds enhanced by RET.

The glycemic regulation attributed to metformin is due to the decrease in hepatic glucose production, increased liver sensitivity to insulin and increased glucose uptake in skeletal muscle, which, in this case, may have been enhanced by RET (glucose uptake independent of insulin) (SBD 2017).
Insulin is essential for regulating the metabolism of carbohydrates and lipids because it reduces the flow of fatty acids from adipose tissue into the circulation through the inhibition of hormone-sensitive lipase and modulates cholesterol synthesis via HMG-CoA reductase. Therefore, absence and/or reduced production/secretion of this hormone causes changes in lipid metabolism (Vergès 2009), as observed in the present study, i.e., increase in triglyceride concentrations in groups of diabetic animals.

In experimental diabetes, Dean and Durrington (1996) observed hypertriglyceridemia in animals with alloxan-induced diabetes as in the present study. Corroborating the findings in experimental models, high LDL concentration and hypertriglyceridemia (Vergès 2009) and reduction in HDL concentrations were found in patients with uncontrolled DM 1 (Mona et al. 2015).

However, treatment with *C. spicata* associated with RET exerted a positive control on the lipid/lipoprotein profile of diabetic animals. The animals of the SD group presented severe and decompensated diabetes during the experimental period. This can be demonstrated by cholesterol concentrations and reduced fractions when compared to the SC group as well as by severe weight loss (Figure 2A) and glycemia above 500 mg/dL (Figure 2B). Therefore, there were animals that presented a compromise in the maintenance of the expected cholesterol concentrations (SC group), which was maintained by the training (TD).

Our results corroborate the hypolipidemic effects of chemical constituents present in species of the Gentianaceae family, mainly swertiamarin, already demonstrated in streptozotocin-induced type-2 diabetic animals (Vaidya et al. 2012) and poloxamer-407-induced hyperlipidemic rats (Vaidya et al. 2009a, 2009b). Such effects may be explained by the ability of swertiamarin to increase the hepatic expression of LDL receptors, inhibit cholesterol synthesis by blocking HMG-CoA reductase and increase HDL concentrations, thus demonstrating its cardioprotector effects, especially in DM (Leong et al. 2016).

Hyperglycemia due to DM 1 is related to the low cellular and tissue energy substrate uptake, generating suppression of cellular ATP genesis, with consequent activation of ROS formation pathways (Shah and Brownlee 2016; Ighodaro 2018). In the incessant search for life, secondary pathways for obtaining energy, such as β-oxidation, which occurs in the mitochondria, are recruited. However, β-oxidation is associated with lipotoxicity, contributing to higher tissue oxidative stress (Jevrić-Causevic et al. 2006). This may explain the increase in serum MDA and SH concentrations observed in diabetic animals (SD and TD) (Table 3).
Natural products have been explored as novel therapeutic targets for the treatment of DM, not only by attenuating hyperglycemia, but also by reducing oxidative stress. In this context, flavonoids stand out for their direct antioxidant properties and for modulating ROS formation pathways and reactive nitrogen species (RNS) (Havsteen 2002). In fact, flavonoids can reduce oxidative damage by decreasing the NADPH oxidase enzyme activity. Hydroxyls contained in the phenolic B ring of flavonoids bind to the C1B regulatory domain of kinase C protein, thereby preventing phosphorylation of the p47fox regulatory domain of NADPH oxidase, decreasing its activity (Yu et al. 2014).

Studies have shown the relationship of RET with oxidative stress and tissue damage, including increased CK, LDH and MDA concentrations (Peternelj and Coombes 2011; Santos et al. 2014). However, the benefits of exercise are related to type, intensity, and duration. Therefore, resistance exercise training of moderate and regular intensity has beneficial effects on oxidative stress and health associated with an adaptive response (Pingitore et al. 2015).

In our study, diabetic animals trained and treated with *C. spicata* or metformin presented reduction in CK, LDH and MDA concentrations when compared to TD, attenuating muscular and oxidative damages when associated with RET, thus demonstrating their protective effects.

Corroborating these results, our research group recently demonstrated the efficiency of treatment with the hydroethanolic extract of *Bowdichia virgilioides* leaves, rich in polyphenols, in attenuating oxidative stress and tissue injury, reducing the MDA and CK concentrations in the muscle and blood tissue of animals submitted to the same RET protocol suggested by Tamaki et al. (1992) (Santos et al. 2014). These results show that treatment with extracts, fractions or antioxidant compounds associated with regular physical exercise can reduce oxidative stress (Haleagrahara et al. 2009; Peternelj and Coombes 2011; Herrlinger et al. 2015).

Due to the fact that DM is a disease that causes damage in different tissues, the present study evaluated the effects of *C. spicata* and RET on target organs such as pancreas, liver, heart and skeletal muscle.

Alloxan is a widely known DM 1 inducer, which induces selective death/necrosis of pancreatic β cells, compromising insulin production. The death mechanism triggered by alloxan is due to its similarity to the glucose molecule and sensitization of GLUT2 transporters and, once recognized, is easily metabolized by the pancreas, which generates a toxic action mediated by increased ROS production and depletion of antioxidant molecules.
such as NADPH and GSH. The latter actively participates in the conversion of alloxan to dialuric acid (Szkudelski 2001; Behr et al. 2008; Lenzen 2008; Silva and Nogueira 2015). In addition, pancreas has less resistance to oxidative stress than other tissues due to its relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase, which makes this tissue more sensitive to the action of ROS (Tiedge et al. 1997; Kajimoto and Kaneto 2004).

These events occur with inhibition of the glycokinase enzyme, a limiting step in intracellular calcium homeostasis, which, at high concentrations in the intracellular environment, leads to high degree of oxidative stress (Iranloye et al. 2011; Dhanesha et al. 2012). However, as a way to compensate for these damages, such as increased MDA observed in diabetic animals (SD and TD), pancreas via Nrf2, transcription factor of cell survival, increases the synthesis of sulfhydryl groups (SH) (Figure 3) (Rohilla and Ali 2012). In contrast, treatment with *C. spicata* prevented the increase of MDA and SH by positively modulating oxidative stress in this tissue.

Nrf2 is a transcription factor activated by oxidative stress and regulates the expression of numerous cell survival genes, such as the antioxidant and detoxifying genes of ROS. Under oxidative stress conditions, Nrf2 increases the expression of genes involved in the synthesis of GSH (glutamate cysteine ligase - GCL) to keep the concentrations of this substrate increased and thus protect cells from oxidative damage. Elevated GSH concentrations may be associated with increased Nrf2 transcriptional activity, which is induced by hyperglycemia (Johnson et al. 2008). This mechanism has also been described for flavonoids (Zhu et al. 2012).

In the hepatic tissue, *C. spicata* extract and fraction did not present significant control over lipoperoxidation, unlike treatment with metformin (Figure 4). In a study carried out by Jaishree and Badami (2010), the isolated active compound, swertiamarin, demonstrated antioxidant effect on the hepatic tissue. Probably, in this tissue, the isolated application promotes high redox-protective activity. However, SH concentrations were lower in TE and TF groups than in control groups (TD and SD). The results found for SH reinforce the evidence of the antioxidant potential of *C. spicata*, more precisely due the presence of swertiamarin. Reduced MDA and SH concentrations in SC and TC groups compared to the other experimental groups confirm the damaging potential of DM 1 on liver tissue and impairment of endogenous antioxidant capacity. In addition, they reinforce the role of *C. spicata* and RET in glycemic control and, consequently, to attenuate oxidative stress mediated by hyperglycemia.
DM has been associated with several cardiovascular complications mediated by oxidative and inflammatory events. In this context, phenolic and secoridoid compounds could act as redox-protective substances to the myocardium and vascular endothelium, inhibiting the formation of reactive species and reducing damages attributed to them (Couto et al. 2018; Delbin et al. 2012; Vaijanathappa et al. 2008; Schnabel and Blankenberg 2007). Although these protective effects were expected for C. spicata, reduction in MDA concentration was not observed in the cardiac tissue, showing that C. spicata did not protect this tissue from damage caused by oxidative stress in DM (Figure 5).

The cascade of signal transduction events involved in the action of insulin is activated by the binding of insulin to the α subunit of its receptor, triggering the autophosphorylation of receptor β subunit tyrosine residues that catalyze the phosphorylation of members of insulin receptor substrates, IRS1 and 2, which culminate in the activation of other intracellular proteins such as Akt and, finally, GLUT4 translocation into the cell membrane surface (Ryder et al. 2001).

Diabetic animals treated with C. spicata extract or fraction and submitted to moderate RET, predominantly anaerobic, presented lower blood glucose concentration, showing the importance of exercise type and intensity. In this context, muscle contraction may increase glucose uptake by activating the insulin-mediated Akt pathway or AMPK activated by ROS and by intracellular calcium concentrations. These findings reveal the participation of skeletal muscle in glycemic control, corresponding to approximately 80-85% of glucose uptake (Santos et al. 2017).

In muscle tissue, diabetic animals showed higher MDA and SH concentrations (Figure 6). In contrast, TE and TF groups showed better redox state, confirming the antioxidant effects of C. spicata. It is important to emphasize that antioxidant properties associated with species of the same family have already been confirmed by other studies (Leong et al. 2016; Wu et al. 2017), which strengthens our findings.

The results found in this study suggest that the muscle and pancreas are the tissues most sensitive to the action of C. spicata and RET, synergistically contributing in glycemic control, weight recovery and attenuation of the deleterious effects associated with oxidative stress caused by the hyperglycemic condition.

Although positive effects of C. spicata and RET on glycemic control and evaluated markers have been demonstrated, some limitations should be considered in this study as the determination of insulin concentration. We believe that these effects may be associated with cytoprotective effects on the pancreas and improved muscle uptake of glucose via exercise-
stimulated insulin. Thus, future studies are necessary to should be done to clarify the effects of *C. spicata* on insulin secretion and/or their sensitivity in different tissues.

5. Conclusion

The results obtained suggest that the medicinal use of *C. spicata* as an anti-hyperglycemic agent and demonstrate its lipid-lowering and antioxidant properties, attenuating oxidative stress induced by DM 1. Thus, *C. spicata* is a potential alternative for the development of therapeutic products for the treatment of DM.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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Table 1. Experimental groups.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TREATMENT</th>
<th>TRAINING</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>Vehicle (distilled water - 0.5mL/kg, po)</td>
<td>Electrostimulation only</td>
</tr>
<tr>
<td>SD</td>
<td>Vehicle (distilled water - 0.5mL/kg, po)</td>
<td>Electrostimulation only</td>
</tr>
<tr>
<td>TC</td>
<td>Vehicle (distilled water - 0.5mL/kg, po)</td>
<td>Electrostimulation + RET</td>
</tr>
<tr>
<td>TD</td>
<td>Vehicle (distilled water - 0.5mL/kg, po)</td>
<td>Electrostimulation + RET</td>
</tr>
<tr>
<td>TE</td>
<td>Extract (100 mg/kg - 0.5 mL/kg, po)</td>
<td>Electrostimulation + RET</td>
</tr>
<tr>
<td>TF</td>
<td>Fraction (100 mg/kg - 0.5 mL/kg, po)</td>
<td>Electrostimulation + RET</td>
</tr>
<tr>
<td>TM</td>
<td>Metformin (100 mg/kg - 0.5 mL/kg, po)</td>
<td>Electrostimulation + RET</td>
</tr>
</tbody>
</table>

Legend: Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM), Resistance Exercise Training Program (RET).

Table 2. Weathered physical training protocol.

<table>
<thead>
<tr>
<th>Weeks of RET</th>
<th>Intensity of RET (%)</th>
<th>Days of the week*</th>
<th>N° of Series</th>
<th>N° of Repetitions</th>
<th>Rest interval (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ª</td>
<td>65</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>2ª</td>
<td>65</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>3ª</td>
<td>65</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>4ª</td>
<td>65</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>60</td>
</tr>
</tbody>
</table>

Model proposed by Tamaki et al. (1992) and adapted by Santos et al. (2014).

* Every other day.
Table 3. Seric biochemical, muscle injury, and oxidative stress markers in alloxan-induced diabetic rats and submitted or not to resistance training treated with extract or fraction of the *C. spicata*, or metformin after 30 days (*n*=08).

<table>
<thead>
<tr>
<th>Groups</th>
<th>SC</th>
<th>SD</th>
<th>TC</th>
<th>TD</th>
<th>TE</th>
<th>TF</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>63.57 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>251.80 ± 33.39&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>225.30 ± 47.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>368.20 ± 19.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>232.60 ± 18.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>289.20 ± 28.76&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>198.40 ± 23.86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDH</td>
<td>238.50 ± 13.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>283.70 ± 42.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>756.20 ± 26.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>726.20 ± 87.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>369.30 ± 27.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>228.40 ± 41.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>233.70 ± 23.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALT</td>
<td>40.16 ± 2.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.23 ± 9.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.84 ± 3.48&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>68.39 ± 8.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.59 ± 5.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.14 ± 2.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.67 ± 11.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST</td>
<td>67.66 ± 2.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140.60 ± 10.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.79 ± 2.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>130.40 ± 30.82&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>111.70 ± 10.88&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>105.20 ± 11.59&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>98.94 ± 8.65&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>2.99 ± 0.10&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>3.83 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.17 ± 0.10&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>3.78 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.80 ± 0.06&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.10 ± 0.12&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>3.10 ± 0.14&lt;sup&gt;ac&lt;/sup&gt;</td>
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<tr>
<td>Lipidic profile (mg/dL)</td>
<td></td>
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<td></td>
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<tr>
<td>Total cholesterol</td>
<td>373.10 ± 25.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>167.80 ± 8.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.61 ± 8.88&lt;sup&gt;c&lt;/sup&gt;</td>
<td>370.90 ± 37.46&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>76.82 ± 4.95&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>126.80 ± 16.10&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>313.50 ± 16.64&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>63.88 ± 4.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.57 ± 1.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.25 ± 1.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>45.39 ± 6.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.46 ± 2.99&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>35.81 ± 0.99&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>33.87 ± 2.72&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td>LDL cholesterol</td>
<td>297.60 ± 30.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120.50 ± 9.37&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>29.02 ± 5.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>257.00 ± 34.96&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>75.47 ± 25.75&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>104.90 ± 8.83&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>180.80 ± 29.14&lt;sup&gt;cd&lt;/sup&gt;</td>
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<tr>
<td>Tryglicerides</td>
<td>156.70 ± 3.12&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>202.50 ± 12.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>134.60 ± 4.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>187.80 ± 7.68&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>187.60 ± 3.25&lt;sup&gt;abd&lt;/sup&gt;</td>
<td>163.40 ± 2.69&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>185.00 ± 5.43&lt;sup&gt;abd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MDA (nmol/mL)</td>
<td>80.92 ± 4.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>318.00 ± 19.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.30 ± 5.39&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>282.10 ± 8.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>140.10 ± 12.68&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>170.00 ± 16.73&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>188.40 ± 9.91&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH (nmol/mL)</td>
<td>68.69 ± 7.32&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>126.60 ± 11.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.86 ± 0.20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>136.90 ± 7.10&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>63.02 ± 11.56&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>32.30 ± 7.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>112.00 ± 12.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FRAP (M Fe produced/mg of protein)</td>
<td>58.92 ± 8.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104.32 ± 16.54&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>62.77 ± 7.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>106.66 ± 14.48&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>119.93 ± 30.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.62 ± 13.82&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>98.43 ± 23.53&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Legend: Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM). The results are expressed mean ± SEM. Different letters indicate significant difference between groups (One-way ANOVA and Bonferroni post hoc).
FIGURE CAPTIONS

Figure 1: Experimental design.

Figura 2. Effects of the C. spicata on weight and blood glucose in alloxan-induced diabetic rats and submitted to resistance exercise. Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM). The results are expressed mean ± SEM. The symbols indicate significant difference (repeated measures ANOVA followed by Bonferroni post-test) *p<0.05 SD vs SC; #p<0.05 TD vs TC; †p<0.05 TE, TF or TM vs TD; δp<0.05 TD, TE, TF or TM vs SD. Different letters on the same line indicate significant difference.

Figura 3. Effects of the C. spicata on oxidative stress markers of pancreatic tissue in alloxan-induced diabetic rats and submitted to resistance training. Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM). The results are expressed mean ± SEM. Different letters significant difference between groups (One-way ANOVA and Bonferroni post hoc).

Figura 4. Effects of the C. spicata on oxidative stress markers of hepatic tissue in alloxan-induced diabetic rats and submitted to resistance training. Legend: Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM). The results are expressed mean ± SEM. Different letters significant difference between groups (One-way ANOVA and Bonferroni post hoc).

Figura 5. Effects of the C. spicata on oxidative stress markers of cardiac tissue in alloxan-induced diabetic rats and submitted to resistance training. Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM). The results are expressed mean ± SEM. Different letters significant difference between groups (One-way ANOVA and Bonferroni post hoc).

Figura 6. Effects of the C. spicata on oxidative stress markers of muscle tissue in alloxan-induced diabetic rats and submitted to resistance training. Sedentary Control (SC), Sedentary Diabetic (SD), Trained Control (TC), Trained Diabetic (TD), Trained Diabetic + Extract (TE), Trained Diabetic + Ethyl Acetate Fraction (TF), Trained Diabetic + Metformin (TM). The results are expressed mean ± SEM. Different letters significant difference between groups (Two-way ANOVA and Bonferroni post hoc).
First week
Adaptation

Diabetes induction

After 72 h
Diabetes confirmation

Day 1-30
Treatment + RET

Euthanasia and sample collection